

PCTWORLD INTELLECTUAL PROPERTY ORGANIZATION
International Bureau

B22

INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(51) International Patent Classification⁵ : A61K 39/00, 39/39, 39/35	A1	(11) International Publication Number: WO 94/27634 (43) International Publication Date: 8 December 1994 (08.12.94)
(21) International Application Number: PCT/AU94/00292 (22) International Filing Date: 1 June 1994 (01.06.94) (30) Priority Data: 08/072,832 2 June 1993 (02.06.93) US (71) Applicant: INSTITUTE FOR CHILD HEALTH RESEARCH [AU/AU]; Princess Margaret Hospital for Children, Roberts Road, Subiaco, W.A. 6008 (AU). (72) Inventor: THOMAS, Wayne, Robert; 31 Taylor Road, Ned- lands, W.A. (AU). (74) Agents: SLATTERY, John, Michael et al.; Davies & Collison, 1 Little Collins Street, Melbourne, VIC 3000 (AU).		(81) Designated States: AT, AU, BB, BG, BR, BY, CA, CH, CN, CZ, DE, DK, ES, FI, GB, GE, HU, JP, KG, KP, KR, KZ, LK, LU, LV, MD, MG, MN, MW, NL, NO, NZ, PL, PT, RO, RU, SD, SE, SI, SK, TJ, TT, UA, UZ, VN, European patent (AT, BE, CH, DE, DK, ES, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, ML, MR, NE, SN, TD, TG). Published <i>With international search report.</i>
(54) Title: CRYPTIC PEPTIDES FOR USE IN INDUCING IMMUNOLOGIC TOLERANCE (57) Abstract Methods of inducing immunologic tolerance in a subject, such as human, by administering a tolerizing amount of a composition comprising a cryptic peptide derived from the antigen and a pharmaceutically acceptable carrier are described. Compositions which include a cryptic peptide derived from a protein antigen, such as an allergen or autoantigen, can be administered to induce tolerance in a naive or pre-sensitized individual. Preferably, the composition is administered orally.		

FOR THE PURPOSES OF INFORMATION ONLY

Codes used to identify States party to the PCT on the front pages of pamphlets publishing international applications under the PCT.

AT	Austria	GB	United Kingdom	MR	Mauritania
AU	Australia	GE	Georgia	MW	Malawi
BB	Barbados	GN	Guinea	NE	Niger
BE	Belgium	GR	Greece	NL	Netherlands
BF	Burkina Faso	HU	Hungary	NO	Norway
BG	Bulgaria	IE	Ireland	NZ	New Zealand
BJ	Benin	IT	Italy	PL	Poland
BR	Brazil	JP	Japan	PT	Portugal
BY	Belarus	KE	Kenya	RO	Romania
CA	Canada	KG	Kyrgyzstan	RU	Russian Federation
CF	Central African Republic	KP	Democratic People's Republic of Korea	SD	Sudan
CG	Congo	KR	Republic of Korea	SE	Sweden
CH	Switzerland	KZ	Kazakhstan	SI	Slovenia
CI	Côte d'Ivoire	LI	Liechtenstein	SK	Slovakia
CM	Cameroon	LK	Sri Lanka	SN	Senegal
CN	China	LU	Luxembourg	TD	Chad
CS	Czechoslovakia	LV	Latvia	TG	Togo
CZ	Czech Republic	MC	Monaco	TJ	Tajikistan
DE	Germany	MD	Republic of Moldova	TT	Trinidad and Tobago
DK	Denmark	MG	Madagascar	UA	Ukraine
ES	Spain	ML	Mali	US	United States of America
FI	Finland	MN	Mongolia	UZ	Uzbekistan
FR	France			VN	Viet Nam
GA	Gabon				

CRYPTIC PEPTIDES FOR USE IN INDUCING IMMUNOLOGIC TOLERANCE

Background of the Invention

5 Feeding antigens has been a classical method for inducing immunological unresponsiveness or oral tolerance (Asherson, G. L., et al., (1977), *Cell Immunol.*, 33:145; Asherson, G. L., et al. (1979), *Immunology*, 36:449; Challacombe, S. J., and Tomasi, T.J., (1980), *J. Exp. Med.*, 152:1459; Bruce, M. G., and Ferguson, A. (1986), *Immunology*, 57:627; Mowat, A. M., et al., (1982), *Immunology*, 45:105; and Strobel, S., et al., (1983),
10 *Immunology*, 49:451). Although regarded as being an important physiological response to dietary antigens (Mowat, A. M., (1987), *Immunology Today*, 8:93), it has been suggested that oral tolerance could be used to control aberrant immunological responses such as those found in autoimmune disease, Thompson, H. S. G., and Staines, A. (1990), *Immunol. Today*, 11:396, and allergy.

15 The most extensively studied model system for autoimmune disease has been that of experimental allergic encephalomyelitis (EAE). It has been shown that rats fed a tolerizing dose of myelin basic protein (MBP) prior to sensitization can be protected from an encephalitogenic challenge with MBP (Miller, A. et al., (1991), *J. Exp. Med.*, 174:791; Whitacre, C. C., et al., (1991), *J. Immunol.*, 147:2155; and Miller, A., et al., (1992),
20 *Proc. Natl. Acad. Sci. USA*, 89:421). However, there have been conflicting views as to the mechanisms involved in inducing oral tolerance. For example, Whitacre et al., (1991), *J. Immunol.*, 147:2155, found they were unable to transfer suppression using T cells from tolerized animals, but showed that clonal anergy may be an important mechanism for down-regulating the effector function of CD4⁺ MBP-reactive T cells. Alternatively, Miller, A. et al., (1991), *J. Exp. Med.*, 174:791, have shown that suppression can be transferred to naive
25 recipients who receive CD8⁺ T cells from tolerized animals. These suppressor (Ts) cells through the release of a soluble cytokine were reported to be able to inhibit the *in vitro* response of a MBP-specific CD4⁺ T cell line and could also bring about a by-stander suppression of unrelated T cells (Miller, A., (1991), *cited supra*). The immunoregulatory
30 cytokine released by Ts cells was later defined as TGF- β 1 (Miller, A., et al. (1992) *Proc. Natl. Acad. Sci. USA* 89:421).

Peptides derived from a variety of protein antigens, including bacterial and viral pathogens, autoantigens, allergens and other experimental antigens such as hen egg lysozyme (HEL), ovalbumin (OVA) and lambda repressor (cl) have been examined for the ability to
35 stimulate antigen-specific T cells. A wide size spectrum of peptides has been reported to serve as T cell epitopes. For example, a peptide derived from Hepatitis B surface antigen (HBsAg amino acid residues 19-33) has recently been shown to stimulate T cell responses in a majority of human subjects who had been immunized with a recombinant hepatitis B vaccine (Schad, V.C. et al., (1991) *Seminars in Immunol.*, 3:217-224). A major

mycobacterial antigen 65-kD protein has also been epitope-mapped (Lamb, J.R. et al., (1987) *EMBO J.*, 6(5):1245-1249). T cell epitopes have been identified in the peptides comprised of amino acid residues 112-132 and 437-459 of the 65-kD protein. MBP has also been epitope-mapped in both human (Ota, K. et al., (1990) *Nature*, 346:183-187) and rodent (Zamvil et al., (1986) *Nature*, 324:258-260) systems.

T cell epitopes present in allergenic proteins have very recently been described (O'Hehir, R. et al., (1991) *Ann. Rev. Immunol.*, 9:67-95). Several peptides derived from the house dust mite allergen Der p I have been shown to be T cell-reactive (Thomas, W.R., et al. In Epitopes of Atopic Allergens Proceedings of Workshop from XIV Congress of the European Academy of Allergy and Clinical Immunology, Berlin (Sept. 1989) pp. 77-82; O'Hehir, R.E. (1991) *Annual Review Immunology* 9:67-95 ; Stewart, G.A. et al. In: Epitopes of Atopic Allergens Proceedings of Workshop from XIV Congress of the European Academy of Allergy and Clinical Immunology, Berlin (Sept. 1989) pp. 41-47; and Yessel, H. et al. In: T Cell Activation in Health and Disease: Discrimination Between Immunity and Tolerance, Conference 22-26 (Sept. 1990) Trinity College, Oxford U.K.). A T cell-stimulatory peptide derived from the short ragweed allergen Amb a I (amino acid residues 54-65) has also been reported (Rothbard, J.B. et al., (1988) *Cell*, 52:515-523). Using a panel of T cell clones derived from a rye grass-allergic individual, Perez et al. demonstrated that T cell epitopes are contained within amino acid residues 191-210 of the protein allergen Lol p I (Perez, M. et al., (1990) *J. Biol. Chem.* 265(27):16210-16215 .

Summary of the Invention

This invention pertains to methods of inducing immunologic tolerance to a protein antigen in a subject, such as human, by administering a tolerizing amount of a composition comprising at least one cryptic peptide derived from the antigen and a pharmaceutically acceptable carrier. Compositions which include a cryptic peptide derived from a protein antigen, such as an allergen or autoantigen, can be administered to induce tolerance in a naive or pre-sensitized individual. Preferably, the composition is administered orally to treat sensitivity in an individual to an allergen or autoantigen.

Brief Description of the Drawings

Figure 1 is a graphic representation of the responses of T cells isolated from mice immunized with Der p I and analyzed for response to selected peptides derived from Der p I by tritiated thymidine incorporation.

Figure 2a and *2b* are graphic representations of the responses of T cells isolated from mice immunized with a selected peptide derived from Der p I and analyzed for response to either Der p I protein (panel a) or the appropriate peptide (panel b).

Figure 3 is a schematic representation of the location of T cell epitopes recognized by mice in the Der p I protein sequence where immunodominant epitopes are represented with hatched squares, cryptic epitopes are represented by dotted squares and the absence of epitopes is represented by black squares.

Figure 4 is a graphic representation of the responses of T cells isolated from mice fed with buffer (panel a), peptide GEX p57-130 (panel b), peptide GEX p101-154 (panel c), or recombinant protein, GEX Der p I (1-222) (panel d) followed by immunization with Der p I and analyzed for response to Der p I *in vitro* by IL-3/GM-CSF (panels a-d) or IL-2 production (panels e-h).

Figure 5 is a graphic representation of the responses of T cells isolated from mice fed recombinant protein GEX Der p II (1-129), peptide GEX p101-154, or peptide GEX p188-222 followed by immunization with Der p I and analyzed for response to Der p I (panel a and d), peptide p110-131 (panel b and e), or peptide p78-100 (panel c and f) by IL-3/GM-CSF (panels a-c) or IL-2 production (panels d-f).

Figure 6 is a graphic representation of the responses of T cells isolated from mice fed with either buffer or recombinant fusion peptide (GEX p131-187) followed by immunization with Der p I and analyzed for response to Der p I by IL-2 production.

Detailed Description of the Invention

This invention pertains to methods for inducing immunologic tolerance to a protein antigen in a subject by administering at least one cryptic peptide derived from the antigen. Protein antigens are known to contain certain determinants or epitopes which, upon presentation with a particular class II major histocompatibility (MHC) molecule will activate T cells of a subject upon exposure to the native protein antigen. Rather than the T cell response being limited by the presence of one or two determinants on an antigen, it appears that the T cell response preferentially utilizes a selected number of determinants. Thus, a hierarchy of T cell determinant usage exists for a multideterminant protein antigen. Accordingly, the T cell determinants or epitopes for a particular protein antigen can be divided into categories based on *in vitro* T cell proliferation assays in which protein antigen-primed T cells are cultured with a selected concentration of a peptide derived from the protein antigen and the amount of proliferation by the T cells in response to the peptide is determined by, for example, tritiated thymidine incorporation.

By this assay, a peptide is categorized as comprising an immunodominant T cell epitope if the peptide consistently induces one of the highest T cell proliferative responses in antigen-primed T cells in the subject tested. Relative to an immunodominant epitope, a peptide which comprises a minor T cell epitope recalls *in vitro* T cell proliferation to a more variable and lesser extent. Those peptides which recall T cell proliferation of less than 2 fold

the background level of media alone are categorized as either not comprising a T cell epitope or comprising a cryptic T cell epitope. Cryptic epitopes are those determinants in a protein antigen which, due to processing and presentation of the native protein antigen to the appropriate MHC molecule, are not normally revealed to the immune system. However, a peptide comprising a cryptic epitope is capable of tolerizing T cells, and when a subject is primed with the peptide, T cells obtained from the subject will proliferate *in vitro* in response to the peptide or the protein antigen from which the peptide is derived. Peptides which comprise at least one cryptic epitope derived from a protein antigen are referred to herein as cryptic peptides. To confirm the presence of cryptic epitopes in a peptide categorized by the above-described assay, antigen-primed T cells are cultured *in vitro* in the presence of each peptide separately to establish peptide-reactive T cell lines. A peptide is considered to comprise at least one cryptic epitope if a T cell line can be established with a given peptide and T cells are capable of proliferation upon challenge with the peptide and the protein antigen from which the peptide is derived.

The presence of cryptic epitopes in a protein antigen is due to a lack of exposure of certain epitopes to the immune system which may result from normal processing of the protein antigen which fails to reveal the epitope to the appropriate class II MHC molecule. Alternatively, the end product of antigen processing may be a large fragment which hides the cryptic epitope and hinders access to the MHC molecule or the T cell receptor on T cells specific for the epitope. Additionally, other epitopes on the same protein antigen may compete with the cryptic epitope for binding to the same restriction element or may have a higher affinity and availability for a different restriction element, thus preventing cryptic epitope interaction with MHC.

Cryptic peptides of the invention comprise at least one cryptic epitope derived from a protein antigen (i.e., the peptide comprises at least approximately 7 amino acid residues). Such peptides can comprise as many amino acid residues as desired and preferably comprise at least about 7, more preferably at least about 15, even more preferably at least about 20 and most preferably at least about 25 amino acid residues of a protein antigen. A peptide length of about 20-40 amino acid residues is preferred as increases in length of a peptide may result in difficulty in peptide synthesis as well as retention of an undesirable property (e.g., immunoglobulin binding or enzymatic activity) due to maintenance of conformational similarity between the peptide and the protein antigen, such as an allergen from which it is derived. If desired, the amino acid sequences of one or more peptides can be produced and joined by a linker to increase sensitivity to processing by antigen-presenting cells. Such linker can be any non-epitope amino acid sequence or other appropriate linking or joining agent. For example, two cryptic peptides can be joined or a cryptic peptide and a peptide

comprising an immunodominant or minor epitope derived from the protein antigen can be linked.

Cryptic peptides can be produced by recombinant DNA techniques in a host cell transformed with a nucleic acid vector directing expression of a nucleotide sequence coding for such peptide, or by chemical synthesis, or in certain limited situations by chemical cleavage of protein antigen such as an allergen. When produced by recombinant techniques, host cells transformed with nucleic acid vectors directing expression of a nucleotide sequence coding for a peptide are cultured in a medium suitable for the cells. The peptides may be secreted and harvested from a mixture of cells and cell culture medium. Alternatively, the peptide may be retained cytoplasmically and the cells harvested, lysed and the peptide isolated and purified. Peptides can be isolated using techniques known in the art for purifying peptides or proteins including ion-exchange chromatography, gel filtration chromatography, ultrafiltration, electrophoresis, and immunoaffinity purification with antibodies specific for the peptide or the protein antigen from which the peptide is derived, or a portion thereof. The cryptic peptides described herein are isolated such that the peptide is substantially free of cellular material or culture medium when produced by recombinant DNA techniques, or substantially free of chemical precursors or other chemicals when synthesized chemically, or obtained by chemical cleavage of a protein allergen or other protein antigen.

To obtain cryptic peptides of the invention where the T cell epitopes of a protein antigen are unknown or ill-defined, the protein structure of the antigen can be reviewed and the sequence divided into at least two peptide fragments of desired lengths. For example, the protein sequence of a protein antigen can be systematically divided into at least two non-overlapping fragments of desired length or overlapping fragments of desired length. As an illustrative example, the known amino acid sequence of Der p I, a major allergen of Dermatophagoides pteronyssinus having an amino acid sequence of 229 residues (shown in SEQ ID NO:1), can be divided into peptide fragments of about 20-35 amino acid residues in length, with each fragment overlapping with another by about 10 amino acids. To maximize the potential of including T cell epitopes in the peptide fragments, areas of overlap and length of each fragment can be designed to maintain the presence of T cell epitopes predicted using algorithms (Rothbard, J. and Taylor, W.R. (1988) *EMBO J.* 7:93-100; and Berzofsky, J.A. (1989) *Philos. Trans. R. Soc. Lond.* 323:535-544). Preferably, human T cell epitopes within a protein antigen can be predicted using known HLA class II binding specific amino acid residues. The resulting peptide fragments can be produced by recombinant DNA techniques or chemical synthesis.

The peptide fragments derived from a protein antigen are tested to determine those fragments having T cell stimulating activity (i.e., proliferation, lymphokine secretion and/or

induction of T cell anergy/tolerization) and thus comprise at least one T cell epitope. For example, human T cell stimulating activity can be tested by culturing T cells obtained from a subject, such as a human, sensitive to a protein antigen (i.e., a subject which has an immune response to the protein antigen) with a peptide fragment derived from the protein antigen and determining the presence of proliferation by T cells in response to the peptide. The presence of proliferation by T cells can be determined by, for example, uptake of tritiated thymidine.

Immunodominant T cell epitopes, minor T cell epitopes and cryptic epitopes can be identified as described in Example 3. To confirm the presence of a cryptic epitope in a selected peptide, T cells are obtained from an individual sensitive to the protein antigen and cultured with each of the cryptic peptides separately to establish peptide-reactive T cell lines. The presence of T cell proliferation or induction of T cell tolerance in response to the peptide and the protein antigen from which the peptide is derived confirms the presence of at least one cryptic epitope in the peptide.

Cryptic peptides of the invention can be derived from a protein antigen such as an allergen or autoantigen. When derived from an allergen, the cryptic peptide can be derived from any known protein allergen, such as an allergen of the following genus: the genus Dermatophagoides; the genus Felis; the genus Ambrosia; the genus Lolium; the genus Cryptomeria; the genus Alternaria; the genus Alder; the genus Betula; the genus Quercus; the genus Olea; the genus Artemisia; the genus Plantago; the genus Parietaria; the genus Canine; the genus Blattella; the genus Apis; the genus Periplaneta; and the genus Sorghum. Cryptic peptides recognized by mice in Der p I, a major allergen of the species Dermatophagoides pteronyssinus, have been determined in mice and comprise amino acid residues 120-143 of Der p I (SEQ ID NO:1), amino acid residues 144-169 of Der p I (SEQ ID NO:1) and amino acid residues 131-187 of Der p I (SEQ ID NO:1).

Cryptic peptides can also be derived from protein antigens other than allergens where immunologic tolerance to an autoantigen is desired. Autoantigens from which cryptic peptides can be derived include insulin, glutamic acid decarboxylase (64K), PM-1 and carboxypeptidase for use in treating diabetes; myelin basic protein for use in treating multiple sclerosis; rh factor for use in treating erythroblastosis fetalis; acetylcholine receptors for use in treating myasthenia gravis; thyroid receptors for use in treating Graves Disease; basement membrane protein for use in treating Good Pasture's syndrome; and thyroid proteins for use in treating thyroiditis.

According to one aspect of this invention, cryptic peptides derived from a protein antigen are administered to a subject to induce immunologic tolerance in the subject to the protein antigen. The term subject includes living organisms capable of mounting an immune response to a protein antigen, e.g., mammals. Examples of subjects include humans, rats, mice, dogs, cats, horses, cows and transgenic species thereof. Immunologic tolerance refers

to a condition in a subject where a block in the development, growth or differentiation of specific lymphocytes in the subject results upon administration of a tolerizing amount of a cryptic peptide of the invention. Tolerance results from the interaction of antigen with antigen receptors on lymphocytes under conditions in which the lymphocytes, instead of becoming activated, are deleted or rendered unresponsive. Tolerance may also be due to the action of specific T or B lymphocytes or other regulatory mechanisms that prevent lymphocyte activation. One mechanism for inhibiting an immune response is the stimulation of a class of lymphocytes, called suppresser T cells, whose principal function is to suppress the activation of specific T and B lymphocytes. In this situation, inhibition is mediated not by the antigen itself but by regulatory cells that are induced by the antigen. Another proposed mechanism for tolerance is a response by the immune system to antigen in which unique or idiotypic determinants of lymphocytes or antibodies specific for the antigen are targeted. This response results in a network of complementary idiotypes and anti-idiotypes which block the stimulation of antigen-specific cells. Finally, the products of activation of B and T lymphocytes, namely antibodies and cytokines, respectively, are themselves capable of regulating specific immunity to result in tolerance in addition to functioning as the principle effector molecules of lymphocytes.

In order to induce immunologic tolerance in a subject, a tolerizing amount of a cryptic peptide derived from a protein antigen is administered to the subject. A tolerizing amount is defined as a dosage of cryptic peptide necessary to induce immunologic tolerance in a subject, such as a human to the antigen from which the cryptic peptide is derived. Immunologic tolerance in a subject is indicated by non-responsiveness or diminution in symptoms to the protein antigen, such as an allergen or autoantigen, as determined by standard clinical procedures (see e.g., Varney et al., (1990) British Medical Journal 302:265-269). When tolerance to an allergen is sought, such non-responsiveness includes diminution in allergen induced allergic symptoms. As referred to herein, a diminution in symptoms to an allergen includes any reduction in the allergic response of a subject, such as a human, to the allergen following a treatment regimen with a cryptic peptide as described herein. This diminution in symptoms may be determined subjectively in a human (e.g., the patient feels more comfortable upon exposure to the allergen), or clinically, such as with a standard skin test.

Cryptic peptides derived from a protein antigen are typically administered to a subject in the form of a composition which includes a pharmaceutically acceptable carrier or diluent. Administration of a composition of the present invention to induce immunologic tolerance in a subject to a protein antigen can be carried out using known procedures, at dosages and for periods of time effective to tolerize the subject to the protein antigen. Effective amounts of the composition will vary according to factors such as the degree of sensitivity of the subject

to the antigen, the age, sex, and weight of the subject, and the ability of the cryptic peptide(s) to induce tolerance in the subject. Dosage regima may be adjusted to provide the optimum therapeutic response. For example, several divided doses may be administered daily or the dose may be proportionally reduced as indicated by the exigencies of the therapeutic situation.

Cryptic peptides may be administered to a subject in a convenient manner such as by injection (subcutaneous, intravenous, etc.), oral administration, inhalation, intranasal, transdermal application, or rectal administration. Preferred routes of administration to induce immunologic tolerance in a subject are oral and intranasal administration. See O'Hehir, R.E. et al. (1993) *Eur. J. Clin. Invest.* 23(12): 763-772). Depending on the route of administration, the active compound (i.e., the cryptic peptide) may be coated with in a material to protect the compound from the action of enzymes, acids and other natural conditions which may inactivate the compound.

To administer a cryptic peptide or peptides by enteral administration, it may be necessary to coat the peptide with, or co-administer the peptide with, a material to prevent its inactivation. For example, the cryptic peptide may be administered to a subject in an appropriate diluent, co-administered with enzyme inhibitors or in an appropriate carrier such as liposomes. Pharmaceutically acceptable diluents include saline and aqueous buffer solutions. Enzyme inhibitors include pancreatic trypsin inhibitor, diisopropylfluorophosphate (DEP) and trasylol. Liposomes include water-in-oil-in-water CGF emulsions as well as conventional liposomes (Strejan et al., (1984) *J. Neuroimmunol.* 7:27). For purposes of inducing tolerance, the composition is preferably administered in non-immunogenic form, e.g., one that does not contain adjuvant.

The active compound may also be administered parenterally. Dispersions can also be prepared in glycerol, liquid polyethylene glycols, and mixtures thereof and in oils. Under ordinary conditions of storage and use, these preparations may contain a preservative to prevent the growth of microorganisms.

Pharmaceutical compositions suitable for injectable use include sterile aqueous solutions (where water soluble) or dispersions and sterile powders for the extemporaneous preparation of sterile injectable solutions or dispersion. In all cases, the composition must be sterile and must be fluid to the extent that easy syringability exists. It must be stable under the conditions of manufacture and storage and must be preserved against the contaminating action of microorganisms such as bacteria and fungi. The carrier can be a solvent or dispersion medium containing, for example, water, ethanol, polyol (for example, glycerol, propylene glycol, and liquid polyethylene glycol, and the like), suitable mixtures thereof, and vegetable oils. The proper fluidity can be maintained, for example, by the use of a coating such as licithin, by the maintenance of the required particle size in the case of

dispersion and by the use of surfactants. Prevention of the action of microorganisms can be achieved by various antibacterial and antifungal agents, for example, parabens, chlorobutanol, phenol, asorbic acid, thimerosal, and the like. In many cases, it will be preferable to include isotonic agents, for example, sugars, polyalcohols such as manitol, sorbitol, sodium chloride in the composition. Prolonged absorption of the injectable compositions can be brought about by including in the composition an agent which delays absorption, for example, aluminum monostearate and gelatin.

Sterile injectable solutions can be prepared by incorporating active compound in the required amount in an appropriate solvent with one or a combination of ingredients enumerated above, as required, followed by filtered sterilization. Generally, dispersions are prepared by incorporating the active compound into a sterile vehicle which contains a basic dispersion medium and the required other ingredients from those enumerated above. In the case of sterile powders for the preparation of sterile injectable solutions, the preferred methods of preparation are vacuum drying and freeze-drying which yields a powder of the active ingredient (i.e., a peptide of the invention) plus any additional desired ingredient from a previously sterile-filtered solution thereof.

When a cryptic peptide or peptides as herein described is suitably protected, as described above, the peptide may be orally administered, for example, with an inert diluent or an assimilable edible carrier. The peptide and other ingredients may also be enclosed in a hard or soft shell gelatin capsule, compressed into tablets, or incorporated directly into the individual's diet. For oral administration, the active compound may be incorporated with excipients and used in the form of ingestible tablets, buccal tablets, troches, capsules, elixirs, suspensions, syrups, wafers, and the like. Such compositions and preparations should contain at least 1% by weight of active compound. The percentage of the compositions and preparations may, of course, be varied and may conveniently be between about 5 to about 80% of the weight of the unit. The amount of active compound in such compositions is such that a suitable dosage will be obtained. Preferred compositions or preparations according to the present invention are prepared so that an oral dosage unit contains between from about 10 mg to about 200 mg of active compound.

As used herein "pharmaceutically acceptable carrier" includes any and all solvents, dispersion media, coatings, antibacterial and antifungal agents, isotonic and absorption delaying agents, and the like. The use of such media and agents for pharmaceutically active substances is well known in the art. Except insofar as any conventional media or agent is incompatible with the active compound, use thereof in the composition is contemplated. Supplementary active compounds can also be incorporated into the compositions.

It may be advantageous to formulate compositions in dosage unit form for ease of administration and uniformity of dosage. Dosage unit form as used herein refers to

physically discrete units suited as unitary dosages for the mammalian subjects to be treated; each unit containing a predetermined quantity of active compound calculated to produce the desired effect in association with the required pharmaceutical carrier. The specification for the dosage unit forms of the invention are dictated by and directly dependent on (a) the
5 unique characteristics of the active compound and the particular effect to be achieved, and (b) the limitations inherent in the art of compounding such an active compound for the treatment of the subject.

Compositions of the invention can include one or more cryptic peptides or a cryptic peptide and a peptide comprising an immunodominant or minor epitope which are
10 administered to subjects, such as humans, who are naive or pre-sensitized to the protein antigen from which the peptide is derived, at dosages and for lengths of time effective to induce tolerance in the subject to the antigen. For example, an amount of one or more of the same or of different compositions effective to induce tolerance in a subject can be administered simultaneously or sequentially. A composition comprising at least two peptides
15 (e.g., a physical mixture of at least two peptides), can also be used in methods of tolerization. For example, a cryptic peptide and a peptide comprising an immunodominant epitope can be co-administered.

The fact that tolerance can be induced by administering a cryptic peptide of the invention (i.e., a peptide which does not contain an epitope recognized during immunization
20 when the entire protein antigen is presented to a subject) is significant. Peptides effective in immunotherapy may therefore not simply be limited to those identified by T-cell clones or polyclonal responses of sensitized individuals. Administration of a cryptic peptide may avoid the potential limitations inherent in administering a peptide containing immunodominant epitopes to sensitized individuals. The use of cryptic peptides also offers the potential for
25 modifying immune responses without having to redirect the development of T-cell clones which have already progressed along T_H1 and T_H2 or equivalent pathways.

It is also possible to modify the structure of cryptic peptides useful in methods of the invention for such purposes as increasing solubility, enhancing therapeutic or preventive efficacy, or stability (e.g., shelf life *ex vivo*, and resistance to proteolytic degradation *in vivo*).
30 A modified peptide can be produced in which the amino acid sequence has been altered, such as by amino acid substitution, deletion, or addition, to modify the ability of the peptide to induce tolerance, or to which a component has been added for the same purpose.

For example, a peptide can be modified so that it maintains the ability to induce T cell anergy or tolerance and bind MHC proteins. In this instance, critical binding residues for the
35 T cell receptor (i.e., the amino acid residues which comprise the cryptic epitope) can be determined using known techniques (e.g., substitution of each residue, such as, for example, with alanine and determination of presence or absence of T cell reactivity). Those residues

shown to be essential can be modified by replacing the essential amino acid with another, preferably similar amino acid residue (a conservative substitution) whose presence is shown to enhance, diminish, but not eliminate or affect T cell reactivity. In addition, those amino acid residues which are not essential for T cell interaction can be modified by being replaced
5 by another amino acid whose incorporation may enhance, diminish or not affect T cell reactivity, but not eliminate binding to relevant MHC. Preferred amino acid substitutions for non-essential amino acids include, but are not limited to substitutions with alanine, glutamic acid or a methyl amino acid.

Another example of a modification of peptides is substitution of cysteine residues
10 preferably with alanine, or glutamic acid, or alternatively with serine or threonine to minimize dimerization via disulfide linkages.

In order to enhance stability and/or reactivity, peptides can also be modified to incorporate one or more polymorphisms in the amino acid sequence of a protein antigen resulting from natural allelic variation. Additionally, D-amino acids, non-natural amino acids
15 or non-amino acid analogues can be substituted or added to produce a modified peptide within the scope of this invention. Furthermore, peptides can be modified using the polyethylene glycol (PEG) method of A. Sehon and co-workers (Wie et al. *supra*) to produce a peptide conjugated with PEG. Modifications of peptides can also include reduction/alkylation (Tarr in: *Methods of Protein Microcharacterization*, J.E. Silver ed.
20 Humana Press, Clifton, NJ, pp 155-194 (1986)); acylation (Tarr, *supra*); esterification (Tarr, *supra*); chemical coupling to an appropriate carrier (Mishell and Shiigi, eds, *Selected Methods in Cellular Immunology*, WH Freeman, San Francisco, CA (1980); U.S. Patent 4,939,239); or mild formalin treatment (Marsh, (1971) *International Archives of Allergy and Applied Immunology* 41: 199-215).

To facilitate purification and potentially increase solubility of peptides, it is possible to add reporter group(s) to the peptide backbone. For example, poly-histidine can be added to a peptide to purify the peptide on immobilized metal ion affinity chromatography (Hochuli, E. et al., (1988) *Bio/Technology*, 6:1321-1235). In addition, specific endoprotease cleavage sites can be introduced, if desired, between a reporter group and amino acid
30 sequences of a peptide to facilitate isolation of peptides free of irrelevant sequences. In order to successfully tolerize a subject to a protein antigen, it may be necessary to increase the solubility of a peptide by adding functional groups to the peptide or by not including hydrophobic regions in the peptide.

To potentially aid proper antigen processing of T cell epitopes within a peptide,
35 canonical protease sensitive sites can be recombinantly or synthetically engineered within the peptide. For example, charged amino acid pairs, such as KK or RR, can be introduced within a peptide during recombinant construction of the peptide. The resulting peptide can be

-12-

rendered sensitive to cathepsin and/or other trypsin-like enzymes cleavage to generate portions of the peptide containing one or more T cell epitopes. In addition, such charged amino acid residues can result in an increase in solubility of a peptide.

Site-directed mutagenesis of DNA encoding a peptide can be used to modify the structure of the peptide. Such methods may involve PCR (Ho et al., (1989) *Gene* 77:51-59) or total synthesis of mutated genes (Hostomsky, Z., et al., (1989) *Biochem. Biophys. Res. Comm.* 161:1056-1063). To enhance bacterial expression, the aforementioned methods can be used in conjunction with other procedures to change the eucaryotic codons in DNA constructs encoding peptides to ones preferentially used in *E. coli*, yeast, mammalian cells or other eucaryotic cells.

This invention is further illustrated by the following non-limiting examples. The contents of all references and published patent applications cited throughout this application are hereby incorporated by reference. The following methodology described in the Materials and Methods section was used throughout the examples set forth below.

MATERIALS AND METHODS

Animals and Antigens

Female B10 and BALB congenic mice, and inbred C57BL/6J were purchased from the Animal Resource Centre, Murdoch, Western Australia at 6-8 weeks of age.

The house dust mite allergen Der p I was affinity purified from spent mite medium (SMM) using previously described techniques (Hoyne, G.F. et al. (1993) *cited supra*; Lombardo et al. *J. Immunol.* 144:1353-1360 and Chapman (1989) *Advances in Biosciences* 74:281-295). Ovalbumin (OVA) crystalline Grade V was purchased from the Sigma Chemical Company, St. Louis, MO. Overlapping synthetic peptides derived from the published Der p I sequence (Chua et al. (1988) *J. Exp. Med.* 167:175-182) were synthesized using standard t-BOC chemistry and peptides were purified by reverse phase high performance liquid chromatography (HPLC) and the sequence of individual peptides were checked to verify identity. The peptides used in this study comprised the following amino acid residues derived from the Der p I sequence (Chua et al. (1988) *cited supra*): 1-20, 13-39, 21-49, 40-60, 50-71, 61-84, 78-100, 85-109, 101-119, 110-131, 120-143, 132-157, 144-169, 158-180, 170-191, 181-204, 197-222.

Preparation of recombinant proteins

Inserts encoding either the whole Der p I or Der p II protein (from spent mite medium, the Commonwealth Serum Laboratories, Melbourne, Australia) or recombinant constructs (formed from the restriction endonuclease fragmentation of the relevant cDNA;

see Chua et al. (1990) *Int. Arch. Allergy Appl. Immunol.* 91:118-123), were ligated to the p-GEX vector and transformed into *Escherichia coli* (Smith, D. B., and Johnson, K. S., (1988) *Gene*, 67:31). The procedures for the molecular cloning of these products have been described elsewhere (Chua, K. Y., et al., (1988) *J. Exp. Med.*, 167:175 and Chua, K. Y., et al., (1990) *Int. Arch. Allergy Appl. Immunol.*, 91:124). Log phase *E. coli* cells transformed with pGEX based protein or peptide constructs were induced to express the recombinant protein by adding 0.1 mM isopropylthiogalactoside (IPTG) (Promega) to the culture with shaking for 60 minutes at 37°C. Because large quantities of fusion peptides were required they were prepared from solubilized inclusions. Bacterial pellets were resuspended in tris buffered saline with 1 mM EDTA and transferred to a homogenizing bottle containing 0.1 mm glass beads and were homogenized using a Braun MSK Homogenizer for five minutes. The lysate was removed after ultracentrifugation at 10,000 g for 10 minutes at 4°C. The pellet was washed twice with 1.75 M guanidine HCL containing 1 M NaCl and 1% triton-X 100 (BDH Chemicals) by thoroughly aspirating in a pipette and then centrifugation. The pellet was then dissolved by incubating it in 8 M urea with 50 mM NaCl and 1 mM ethylene diamine tetraacetic acid (EDTA) for 2 hours at 37°C. The sample was dialyzed in 3-(cyclohexylamino)-propanesulfonic acid (CAPS) buffer pH 10.7 and the pH was slowly adjusted to pH 9.6. The recombinant material was then clarified by centrifugation at 10 000 g and the concentration of the soluble material was estimated against standard quantities of bovine serum albumin (BSA) using SDS-polyacrylamide gel electrophoresis (SDS-PAGE) and staining with Coomassie blue. Recombinant peptides used in this study included GEX Der p I (amino acid residues 1-222), GEX Der p II (amino acid residues 1-129), GEX p1-14 (amino acid residues 1-14 of Der p I), GEX p60-111 (amino acid residues 60-111 of Der p I), GEX p98-140 (amino acid residues 98-140 of Der p I), GEX p101-154 (amino acid residues 101-154 of Der p I), GEX p57-130 (amino acid residues 57-130 of Der p I), GEX p188-222 (amino acid residues 188-222 of Der p I).

Induction of Oral Tolerance

Mice were lightly anesthetized under ether and fed intragastrically by a tube with 3 mg of protein or peptide on 3 consecutive days. Antigens were dissolved in CAPS buffer and administered in a volume of 0.2 ml. Mice were immunized subcutaneously at the base of tail 7 days after the last feed with 100 mg of native protein emulsified in complete Freund's Adjuvant (CFA) in a volume of 0.2 ml.

Culture Medium

Lymph node cells were cultured in Dulbecco's Modified Eagles medium (DME) supplemented with 2% fetal calf serum (FCS), 50 mM, 2-ME, 2 mM L-Glutamine and 20

mg/ml gentamycin. FDC-P1 cells (Kelso, A., (1990), *J. Immunol.*, 145:2167) were maintained in DME + 5% FCS while CTLL-2 cells (Krillis, S. (1978) *J. Immunol.* 120:20) were maintained in Rosewall Park Memorial Institute (RPMI) medium + 10% FCS.

5 T Cell Assays

The periaortic and inguinal lymph nodes were collected from immunized mice and single cell suspensions were prepared by expressing the nodes through a stainless steel wire mesh. Cells were washed and cultured at 4×10^5 cells in a volume of 0.2 ml in DME culture medium in a 96 well flat bottom tissue culture plate. Protein or peptide antigens were added at various concentrations and the cells were incubated at 37°C for 24 hours. Supernatants were collected and stored at -20°C until required. The Der p I used for all *in vitro* assays was the allergen isolated from spent mite medium (SMM).

Lymphokine Assays

15 FDC-P1 cells proliferate maximally in response to IL-3 and GM-CSF and submaximally to IFN- γ or IL-4 (Kelso, A. (1990) *cited supra*). 2×10^3 cells were added in 50 μ l DME + 5% FCS to 50 μ l of culture supernatant in 96 well flat bottom tissue culture plates. The cells were incubated for 40 hours at 37°C and then pulsed with 1 μ Ci 3 H-Thymidine for another 4-6 hours at 37°C. The cells were then harvested onto glass fiber filter mats and samples counted for 3 H-Thymidine incorporation using liquid scintillation spectrometry or for latter experiments due to its acquisition, on a Packard matrix 9600 direct beta counter (Packard Instruments, Meriden, CT).

The CTLL-2 cell line will proliferate maximally with IL-2 but only poorly in the presence of IL-4 (Kelso, A. (1990) *J. Immunol.* 145:2167). Supernatants were cultured with 25 5000 CTLL-2 cells per well for 24 hours at 37°C and pulsed with 1 μ Ci of 3 H-thymidine (3 H-Tdr). Cells were harvested onto glass fiber filter mats and the amount of radioactivity incorporated was determined as described above.

Example 1

Determination of Immunodominant, Minor and Cryptic

30 T Cell Epitopes Recognized by Mice in Der p I

It has been previously shown that H2^b mice are high responders to Der p I while H2^k, H2^d and H2^q mice are low responders (Hoyne, G. (1992) Ph.D. Thesis, T cell Recognition During Mucosal and Systemic Responses, University of Western Australia). To determine the location of T-cell epitopes on Der p I, B10 mice were immunized subcutaneously with 35 100 μ g of Der p I in CFA and after 8 days the periaortic and inguinal lymph nodes were examined for antigen specific lymphokine release (IL-3/GM-CSF) using a panel of overlapping peptides. In three separate experiments the greatest response was found to

peptide p110-131 (amino acid residues 110-131 of Der p I) while lower responses were also seen to peptides p78-100 (amino acid residues 78-100 of Der p I) and p21-49 (amino acid residues 21-49 of Der p I). No other peptides could stimulate a response. An example of the results of one such experiment is shown in Figure 1 in which the following peptides were used: peptide p110-131 (\square); and peptide p78-100 (Δ) and peptide p21-49 (\bullet).

To test for cryptic epitopes, mice were immunized with all the peptides and responses to Der p I and the immunizing peptide were measured in the presence of spleen adherent cells. Peptide p120-143 (amino acid residues 120-143 of Der p I) and peptide p144-169 (amino acid residues 144-169 of Der p I) were able to sensitize mice so they could recall responses to both intact Der p I protein (Figure 2a) and the peptides (Figure 2b) respectively. The results of Figure 2 show the mean IL-3/GM-CSF response of triplicate samples. The following peptides are shown in the Figure: peptide p120-143 (\square); peptide p144-169 (Δ); peptide p132-157 (O); and peptide p158-180 (X).

15 Example 2 Induction of Oral Tolerance in Mice by Administration of Fusion Peptides

A number of recombinant peptides were generated by restriction enzyme digestion of Der p I cDNA. These fragments were cloned into the pGEX expression vector as described above and transformed into E. coli. The recombinant peptides chosen for use in this study were expressed as fusions attached to the glutathione-S-transferase protein of Schistosoma japonicum. The fusion proteins and peptides were solubilized from bacterial cell pellets and dialyzed into CAPS buffer pH 9.6. The recombinant peptides listed in Figure 3 were chosen on the basis of the known T-cell epitope data described above. Recombinant peptides were selected for the presence of immunodominant (hatched squares) or cryptic epitopes (dotted squares) or the absence of T cell epitopes (black squares) within the sequence. Thus, control peptides GEX p1-23 (amino acid residues 1-23 of Der p I) and GEX p188-222 (amino acid residues 197-222 of Der p I) did not contain any T cell epitopes. GEX p57-130 (amino acid residues 57-130 of Der p I) contained two epitopes while GEX p101-154 (amino acid residues 101-154 of Der p I) and GEX p98-140 (amino acid residues 57-130 of Der p I) contains the single immunodominant epitope (amino acid residues 110-131), while GEX p131-187 (amino acid residues 131-187 of Der p I) contains the cryptic epitopes.

Following a previously characterized regime for inducing oral tolerance (Hoyne, G. F., (1993), *Immunology* 78:534-540), mice were fed 3 mg of fusion peptide on 3 consecutive days and after a further 7 days were immunized subcutaneously with native protein in CFA. *In vitro* lymphokine assays were then performed 7 days later using the periaortic and inguinal lymph nodes stimulated with either protein or synthetic peptides. Experiments were performed to show that feeding mice CAPS buffer or the recombinant GEX Der p I (1-222)

fusion protein did not effect the IL-2 or IL-3/GM-CSF responses of mice to subcutaneous injection of OVA in CFA.

To test whether orally administered peptides could induce tolerance, control mice were fed CAPS buffer (Figure 4, panels a and e), while test animals received 3 mg on three consecutive days of either GEX Der p I (1-222) (Figure 4 panels d and h) or the fusion peptides GEX p57-130 (Figure 4, panels b and f) or GEX p101-154 (Figure 4, panels c and g). One week later the response to immunization with native Der p I in CFA was determined. Mice fed CAPS buffer showed strong responses to the Der p I protein *in vitro* secreting both IL-3/GM-CSF (Figure 4, panel a) and IL-2 (Figure 4, panel e) in response to TCR triggering. On the other hand, mice fed GEX Der p I (1-222) or either of the two peptides GEX p57-130 or GEX 101-154 had depressed IL-2 responses (Figure 4, panels f-h). The more pronounced inhibition of IL-2 responses was a consistent feature of all experiments of this nature. Mice were also fed with GEX p98-140 and an equal degree of tolerance induced by this peptide was found.

To examine how the development of oral tolerance effected responses to T cell epitopes on the allergen mice were fed 3 mg on three consecutive days of either GEX p101-154 or GEX p188-222 and GEX Der p II (1-129) as a control. One week later mice were immunized with Der p I and the responses of draining lymph node cells were measured to the protein and peptides *in vitro*. The data shows the response for individual mice in each group at the following antigen concentrations: Der p I, 20 µg/ml; and peptide p110-131 and peptide p78-100, 10 µM. As seen in Figure 5 feeding mice either GEX p188-222 or GEX Der p II (1-129) did not affect the capacity of their lymph node cells to secrete either IL-3/GM-CSF or IL-2 upon *in vitro* challenge with either protein or with the immunogenic peptides p110-131 or p78-100. However, in contrast, the lymphokine responses of GEX p101-154 fed mice were markedly reduced and thus appear to have become tolerant to the whole protein (Figure 5). The tolerance induced by feeding one epitope appears to affect T cells specific for other epitopes on the allergen. Subsequent experiments using GEX p61-100 which contains one epitope and the fusion peptide GEX p1-23 as a control gave the same result.

To determine whether a peptide containing a cryptic epitope could influence the immune response, mice were fed 3mg on three consecutive days of GEX p131-187 (Figure 6 (•)) which contains the cryptic epitope found on peptide 144-169 while control mice were fed with CAPS buffer (Figure 6 (□)). One week later mice were immunized with Der p I in CFA. Lymph node cells were cultured *in vitro* with Der p I and supernatants assayed for IL-2. Each data point in Figure 6 represents the mean response of 5 animals per group ± standard deviation. The responses of cryptic peptide fed mice were statistically different ($p < 0.05$ t-test). As shown in Figure 6 lymph node cells from control mice showed strong

responses to Der p I *in vitro* by secreting IL-2, but mice fed the cryptic epitope displayed much weaker lymphokine response *in vitro*.

The results presented here show that feeding fusion peptides containing dominant or cryptic T-cell epitopes can inhibit T cell responses to subcutaneous immunization with the whole antigen. In the case of fusion peptides containing dominant epitopes the inhibition was profound and was measured by depressed IL-2 and GM-CSF release from draining lymph node cells challenged *in vitro* with whole allergen or the immunodominant peptides. This included responses to peptides containing residues which were not present on the fusion used for feeding. For example, feeding the fusion peptide GEX p101-154 inhibited the ability of Der p I immunization to induce T cells which react with the whole allergen and with synthetic peptides p110-131 and p78-100. This effect may therefore be mediated by a soluble factor. The inhibition was otherwise specific because it could not be induced by the Der p II fusion protein. Similar data has been obtained by Miller, A., et al., (1991), *J. Exp. Med.*, 174:791; Whitacre, C. C., et al., (1991), *J. Immunol.*, 147:2155; and Miller, A., et al., (1992), *Proc. Natl. Acad. Sci. USA*, 89:421, who found that oral tolerance to MBP was mediated by TGF- β 1 and could be shown to suppress bystander responses in an *in vitro* model.

Feeding two fusion proteins that did not contain T-cell epitopes did not inhibit the immune responses. However, feeding the fusion peptide GEX p131-187 which contained the cryptic epitope found in peptide p144-169 did significantly inhibit. The degree of inhibition was not as marked as for the fusions containing dominant epitopes but presumably could be increased by extending the feeding regime or increasing the dose. Feeding the fusion peptides was also found to sensitize T cells in the MLN so they release GM-CSF on stimulation *in vitro* with Der p I or synthetic peptides including the cryptic peptide p144-169 after feeding peptide GEX p131-187. The presence of these sensitized cells in oral tolerance has recently been described for OVA (Hoyne, G. F., et al., (1993), *Immunology* 78:534-540).

Example 3 Determination of Immunodominant, Minor and
Cryptic T Cell Epitopes Recognized by an
Allergic Individual in Der p I

To determine T cell epitopes recognized by an allergic individual in the Der p I protein sequence a T cell line can be established by culturing mite-allergic patient peripheral blood white cells in complete medium at 2×10^6 /ml in the presence of 20 μ g purified native Der p I/ml. After 7 days of culture at 37°C in a humidified CO₂ incubator the viable cells can be isolated by centrifugation with lymphocyte separation medium (LSM, Organon Technica, Durham, NC) and cultured in complete medium containing recombinant IL-2 and recombinant IL-4 for 2-3 additional weeks. When the T cells are "rested" and no longer responsive to growth factors, a secondary proliferation assay can be performed by culturing 2

- x 10⁴ T cells in 200 µl complete medium with 5 x 10⁴ gamma-irradiated (3500 Rads) peripheral white blood cells as antigen presenting cells in the presence of various concentrations of peptides derived from the intact protein. The cultures can then be pulsed with tritiated thymidine (1 µCi/well) on day 3 and harvested onto glass fiber filters on day 4.
- 5 Peptides stimulating tritium incorporation at least 2-fold over the medium control are defined as containing T cell epitopes naturally exposed to the T cells when presented with the entire protein (i.e., the peptides comprise at least one minor or immunodominant epitope). Those peptides stimulating tritium incorporation of less than 2-fold above the medium control either
- 10 do not contain a T cell epitope or contain a cryptic epitope (i.e., an epitope not normally exposed to T cells when the entire protein is presented). To confirm the presence of a cryptic epitope in these peptides, T cell lines can be established by culturing peripheral blood white cells from the same individual in the presence of each peptide separately to establish peptide-reactive T cell lines. The "rested" T cells can then be challenged with each peptide and the Der p I protein. A peptide which comprises at least one cryptic epitope is capable of
- 15 stimulating the proliferation of the T cell line in the presence of the peptide or the entire protein at a level at least 2-fold above the medium control or is capable of tolerizing T cells.

EQUIVALENTS

- 20 Those skilled in the art will recognize, or be able to ascertain using no more than routine experimentation, many equivalents to the specific embodiments of the invention described herein. Such equivalents are intended to be encompassed by the following claims.

SEQUENCE LISTING

(1) GENERAL INFORMATION:

5 (i) APPLICANT:

(A) NAME: INSTITUTE FOR CHILD HEALTH RESEARCH

(B) STREET: P.O. BOX 885

(C) CITY: WEST PERTH

(D) STATE: WESTERN AUSTRALIA

10 (E) COUNTRY: AUSTRALIA

(F) POSTAL CODE (ZIP): 6872

15 (ii) TITLE OF INVENTION: CRYPTIC PEPTIDES FOR USE IN INDUCING
IMMUNOLOGIC TOLERANCE

(iii) NUMBER OF SEQUENCES: 2

(iv) COMPUTER READABLE FORM:

20 (A) MEDIUM TYPE: Floppy disk

(B) COMPUTER: IBM PC compatible

(C) OPERATING SYSTEM: PC-DOS/MS-DOS

(D) SOFTWARE: ASCII TEXT

25 (v) CURRENT APPLICATION DATA:

(A) APPLICATION NUMBER:

(B) FILING DATE:

(vi) PRIOR APPLICATION DATA:

30 (A) APPLICATION NUMBER: US 08/072,832

(B) FILING DATE: 2-JUN-1993

(viii) ATTORNEY/AGENT INFORMATION:

(A) NAME: MANDRAGOURAS, AMY E.

35 (B) REGISTRATION NUMBER: 36,207

(C) REFERENCE/DOCKET NUMBER: IMI-037CPPC

-20-

(ix) TELECOMMUNICATION INFORMATION:

(A) TELEPHONE: (617) 227-7400

(B) TELEFAX: (617) 227-5941

5 (2) INFORMATION FOR SEQ ID NO:1:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 834 base pairs

(B) TYPE: nucleic acid

10 (C) STRANDEDNESS: single

(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

15 (ix) FEATURE:

(A) NAME/KEY: CDS

(B) LOCATION: 1..738

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:1:

20

AAA AAC CGA TTT TTG ATG AGT GCA GAA GCT TTT GAA CAC CTC AAA ACT 48

Lys Asn Arg Phe Leu Met Ser Ala Glu Ala Phe Glu His Leu Lys Thr

1 5 10 15

25 CAA TTC GAT TTG AAT GCT GAA ACT AAC GCC TGC AGT ATC AAT GGA AAT 96

Gln Phe Asp Leu Asn Ala Glu Thr Asn Ala Cys Ser Ile Asn Gly Asn

20 25 30

GCT CCA GCT GAA ATC GAT TTG CGA CAA ATG CGA ACT GTC ACT CCC ATT 144

30 Ala Pro Ala Glu Ile Asp Leu Arg Gln Met Arg Thr Val Thr Pro Ile

35 40 45

CGT ATG CAA GGA GGC TGT GGT TCA TGT TGG GCT TTC TCT GGT GTT GCC 192

Arg Met Gln Gly Gly Cys Gly Ser Cys Trp Ala Phe Ser Gly Val Ala

35 50 55 60

-21-

	GCA ACT GAA TCA GCT TAT TTG GCT CAC CGT AAT CAA TCA TTG GAT CTT	240
	Ala Thr Glu Ser Ala Tyr Leu Ala His Arg Asn Gln Ser Leu Asp Leu	
	65 70 75 80	
5	GCT GAA CAA GAA TTA GTC GAT TGT GCT TCC CAA CAC GGT TGT CAT GGT	288
	Ala Glu Gln Glu Leu Val Asp Cys Ala Ser Gln His Gly Cys His Gly	
	85 90 95	
10	GAT ACC ATT CCA CGT GGT ATT GAA TAC ATC CAA CAT AAT GGT GTC GTC	336
	Asp Thr Ile Pro Arg Gly Ile Glu Tyr Ile Gln His Asn Gly Val Val	
	100 105 110	
15	CAA GAA AGC TAC TAT CGA TAC GTT GCA CGA GAA CAA TCA TGC CGA CGA	384
	Gln Glu Ser Tyr Tyr Arg Tyr Val Ala Arg Glu Gln Ser Cys Arg Arg	
	115 120 125	
20	CCA AAT GCA CAA CGT TTC GGT ATC TCA AAC TAT TGC CAA ATT TAC CCA	432
	Pro Asn Ala Gln Arg Phe Gly Ile Ser Asn Tyr Cys Gln Ile Tyr Pro	
	130 135 140	
25	CCA AAT GCA AAC AAA ATT CGT GAA GCT TTG GCT CAA ACC CAC AGC GCT	480
	Pro Asn Ala Asn Lys Ile Arg Glu Ala Leu Ala Gln Thr His Ser Ala	
	145 150 155 160	
30	ATT GCC GTC ATT ATT GGC ATC AAA GAT TTA GAC GCA TTC CGT CAT TAT	528
	Ile Ala Val Ile Ile Gly Ile Lys Asp Leu Asp Ala Phe Arg His Tyr	
	165 170 175	
35	GAT GGC CGA ACA ATC ATT CAA CGC GAT AAT GGT TAC CAA CCA AAC TAT	576
	Asp Gly Arg Thr Ile Ile Gln Arg Asp Asn Gly Tyr Gln Pro Asn Tyr	
	180 185 190	
40	CAC GCT GTC AAC ATT GTT GGT TAC AGT AAC GCA CAA GGT GTC GAT TAT	624
	His Ala Val Asn Ile Val Gly Tyr Ser Asn Ala Gln Gly Val Asp Tyr	
	195 200 205	

-22-

TGG ATC GTA CGA AAC AGT TGG GAT ACC AAT TGG GGT GAT AAT GGT TAC 672
 Trp Ile Val Arg Asn Ser Trp Asp Thr Asn Trp Gly Asp Asn Gly Tyr
 210 215 220

5 GGT TAT TTT GCT GCC AAC ATC GAT TTG ATG ATG ATT GAA GAA TAT CCA 720
 Gly Tyr Phe Ala Ala Asn Ile Asp Leu Met Met Ile Glu Glu Tyr Pro
 225 230 235 240

TAT GTT GTC ATT CTC TAAACAAAAA GACAATTTCT TATATGATTG TCACTAATTT 775
 10 Tyr Val Val Ile Leu
 245

ATTTAAAATC AAAATTTTTT AGAAAATGAA TAAATTCATT CACAAAATT AAAAAAAAAA 834

15

(2) INFORMATION FOR SEQ ID NO:2:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 245 amino acids
 20 (B) TYPE: amino acid
 (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

25

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:2:

Lys Asn Arg Phe Leu Met Ser Ala Glu Ala Phe Glu His Leu Lys Thr
 1 5 10 15

30 Gln Phe Asp Leu Asn Ala Glu Thr Asn Ala Cys Ser Ile Asn Gly Asn
 20 25 30

Ala Pro Ala Glu Ile Asp Leu Arg Gln Met Arg Thr Val Thr Pro Ile
 35 40 45

35 Arg Met Gln Gly Gly Cys Gly Ser Cys Trp Ala Phe Ser Gly Val Ala
 50 55 60

-23-

Ala Thr Glu Ser Ala Tyr Leu Ala His Arg Asn Gln Ser Leu Asp Leu
65 70 75 80

5 Ala Glu Gln Glu Leu Val Asp Cys Ala Ser Gln His Gly Cys His Gly
85 90 95

Asp Thr Ile Pro Arg Gly Ile Glu Tyr Ile Gln His Asn Gly Val Val
100 105 110

10 —
Gln Glu Ser Tyr Tyr Arg Tyr Val Ala Arg Glu Gln Ser Cys Arg Arg
115 120 125

Pro Asn Ala Gln Arg Phe Gly Ile Ser Asn Tyr Cys Gln Ile Tyr Pro
15 130 135 140

Pro Asn Ala Asn Lys Ile Arg Glu Ala Leu Ala Gln Thr His Ser Ala
145 150 155 160

20 Ile Ala Val Ile Ile Gly Ile Lys Asp Leu Asp Ala Phe Arg His Tyr
165 170 175

Asp Gly Arg Thr Ile Ile Gln Arg Asp Asn Gly Tyr Gln Pro Asn Tyr
180 185 190

25 His Ala Val Asn Ile Val Gly Tyr Ser Asn Ala Gln Gly Val Asp Tyr
195 200 205

Trp Ile Val Arg Asn Ser Trp Asp Thr Asn Trp Gly Asp Asn Gly Tyr
30 210 215 220

Gly Tyr Phe Ala Ala Asn Ile Asp Leu Met Met Ile Glu Glu Tyr Pro
225 230 235 240

35 Tyr Val Val Ile Leu
245

CLAIMS

1. A method of inducing immunologic tolerance in a subject to a protein antigen comprising administering to the subject a tolerizing amount of a composition comprising at least one cryptic peptide derived from the antigen and a pharmaceutically acceptable carrier.
2. A method of claim 1 wherein the subject is a mammal.
3. A method of claim 2 wherein the mammal is a human.
4. A method of claim 1 wherein the composition is administered orally.
5. A method of claim 1 wherein the protein antigen is an allergen.
6. A method of claim 5 wherein the allergen is of a genus selected from the group consisting of: the genus Dermatophagoides; the genus Felis; the genus Ambrosia; the genus Lolium; the genus Cryptomeria; the genus Alternaria; the genus Alder; the genus Betula; the genus Quercus; the genus Olea; the genus Artemisia; the genus Plantago; the genus Parietaria; the genus Canine; the genus Blattella; the genus Apis; the genus Periplaneta; and the genus Sorghum.
7. A method of claim 6 wherein the allergen is of the species Dermatophagoides pteronyssinus.
8. A method of claim 7 wherein the allergen is Der p I.
9. A method of claim 1 wherein the protein antigen is an autoantigen.
10. A method of claim 9 wherein the autoantigen is selected from the group consisting of: insulin; myelin basic protein; rh factor; acetylcholine receptors; thyroid cell receptors; basement membrane proteins; thyroid proteins; PM-1; glutamic acid decarboxylase (64K); and carboxypeptidase H.
11. A method of claim 2 wherein the mammal is a mammal sensitized to the protein antigen.

12. A method of claim 1 wherein the composition further comprises a peptide comprising an immunodominant epitope derived from the protein antigen.

13. A method of inducing immunologic tolerance in a subject to an allergen comprising orally administering to the subject a tolerizing amount of a composition comprising at least one cryptic peptide derived from the allergen and a pharmaceutically acceptable carrier.

14. A method of claim 13 wherein the allergen is of a genus selected from the group consisting of: the genus Dermatophagoides; the genus Felis; the genus Ambrosia; the genus Lolium; the genus Cryptomeria; the genus Alternaria; the genus Alder; the genus Betula; the genus Quercus; the genus Olea; the genus Artemisia; the genus Plantago; the genus Parietaria; the genus Canine; the genus Blattella; the genus Apis; the genus Periplaneta; and the genus Sorghum.

15. A method of claim 14 wherein the allergen is of the species Dermatophagoides pteronyssinus.

16. A method of claim 15 wherein the allergen is Der p 1.

17. A method of claim 13 wherein the subject is a human.

18. A method of claim 17 wherein the subject is a human sensitized to the allergen.

19. A composition for inducing immunologic tolerance in a subject to a protein antigen, the composition comprising a tolerizing amount of at least one cryptic peptide derived from the protein antigen and a pharmaceutically acceptable carrier.

20. A composition of claim 19 in a form suitable for oral administration.

21. A composition of claim 19 wherein the protein antigen is an allergen.

22. A composition of claim 21 wherein the allergen is of a genus selected from the group consisting of: the genus Dermatophagoides; the genus Felis; the genus Ambrosia; the genus Lolium; the genus Cryptomeria; the genus Alternaria; the genus Alder; the genus Betula; the genus Quercus; the genus Olea; the genus Artemisia; the genus Plantago; the

genus Parietaria; the genus Canine; the genus Blattella; the genus Apis; the genus Periplaneta; and the genus Sorghum.

23. A composition of claim 22 wherein the allergen is of the species
5 Dermatophagoides pteronyssinus.

24. A composition of claim 23 wherein the allergen is Der p I.

25. A composition of claim 19 wherein the protein antigen is an autoantigen.
10

26. A composition of claim 25 wherein the autoantigen is selected from the group consisting of: insulin; myelin basic protein; rh factor; acetylcholine receptors; thyroid cell receptors; basement membrane proteins; thyroid proteins; PM-1; glutamic acid decarboxylase (64K); and carboxypeptidase H.
15

27. A composition of claim 19 further comprising a tolerizing amount of a peptide comprising an immunodominant epitope derived from the protein antigen.

28. A composition for inducing oral tolerance in a subject to an allergen, the
20 composition comprising a tolerizing amount of a cryptic peptide derived from the allergen and a pharmaceutically acceptable carrier, in a form suitable for oral administration.

29. A composition of claim 28 further comprising a tolerizing amount of a peptide comprising an immunodominant epitope derived from the protein antigen.

1/6

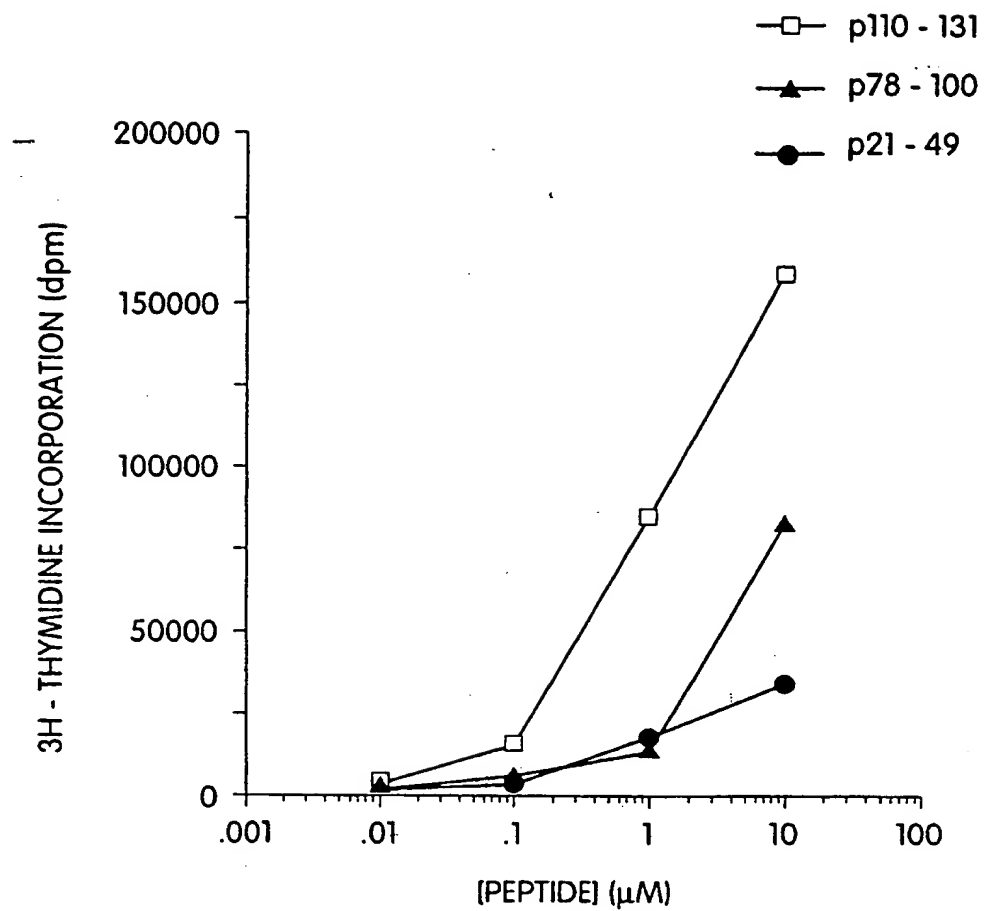


Fig. 1

2/6

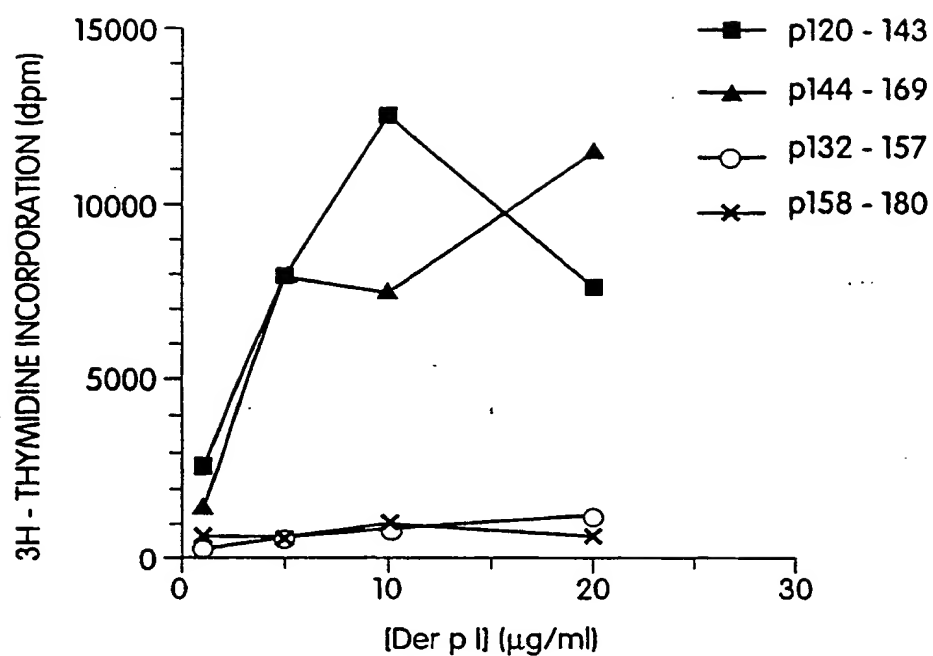


Fig. 2A

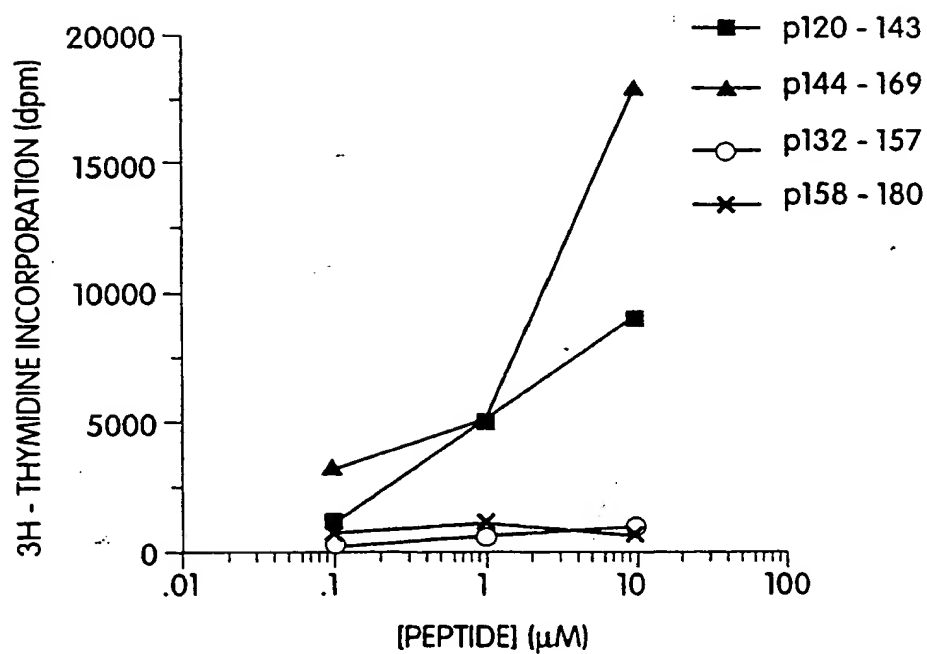


Fig. 2B.

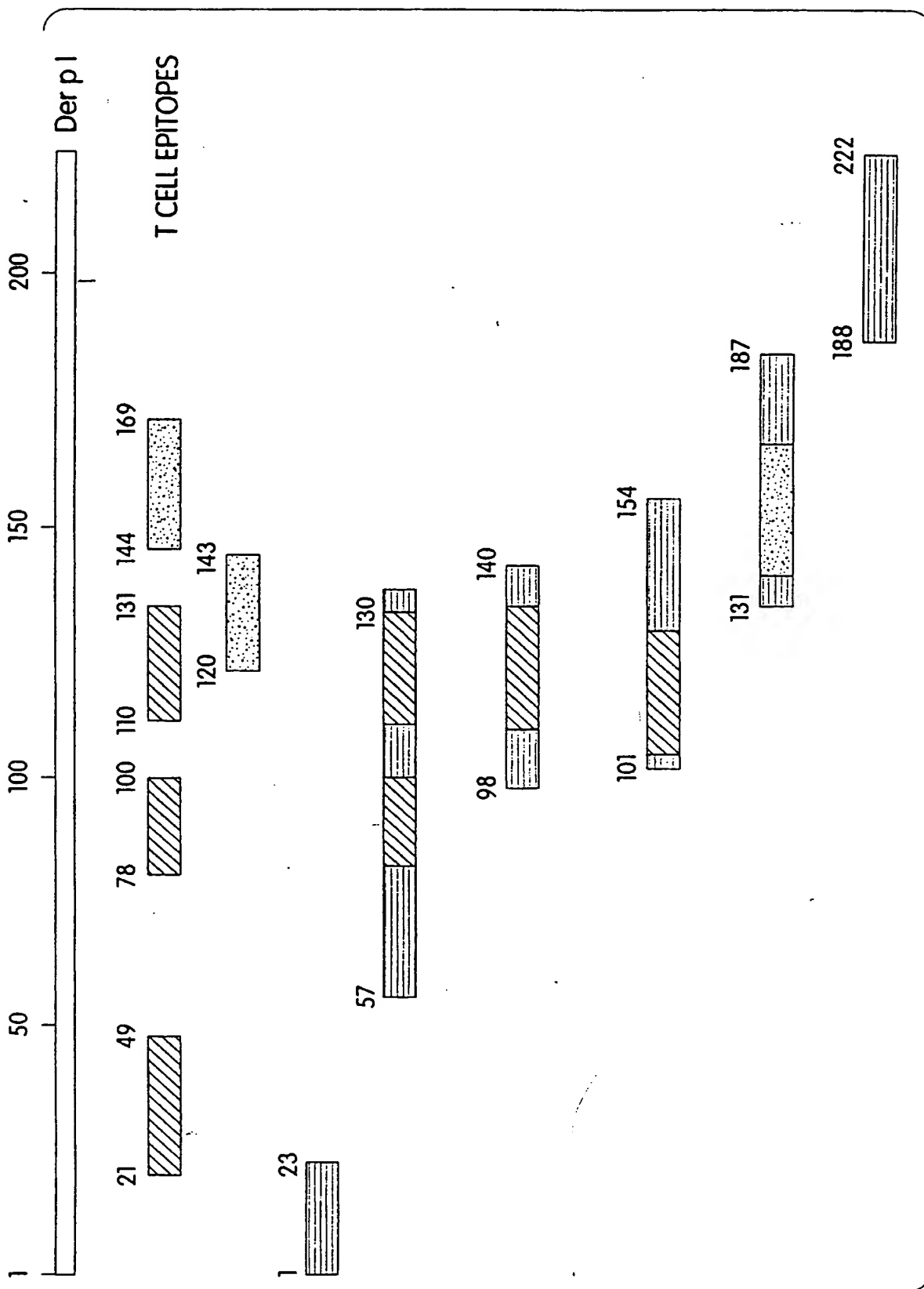


Fig. 3

4/6

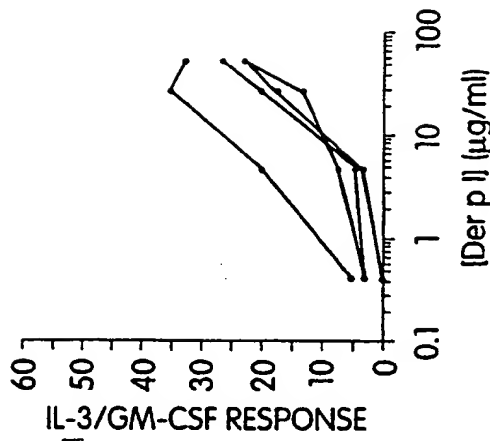


Fig 4D

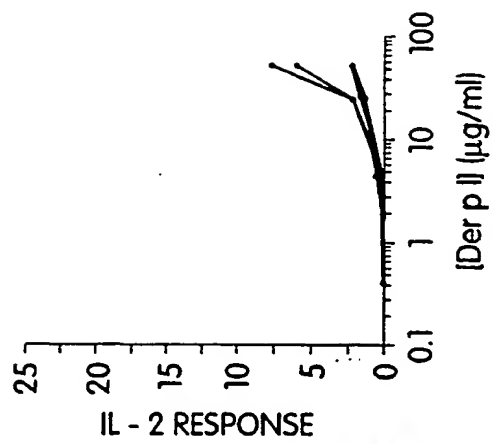


Fig 4H

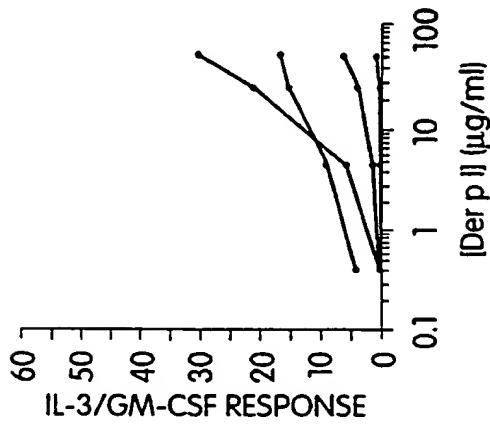


Fig 4C

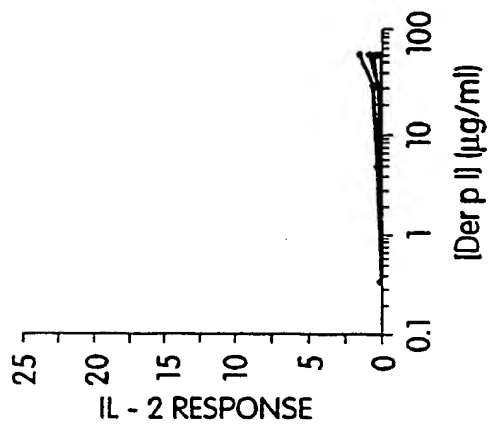


Fig 4G

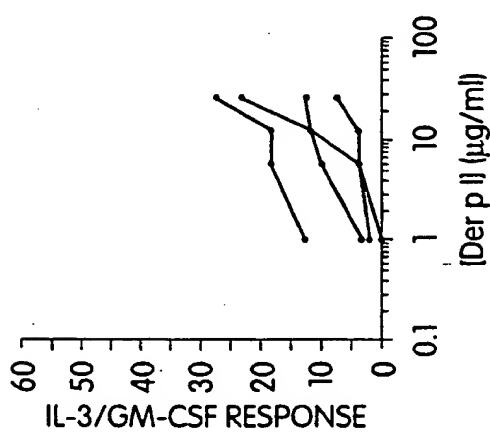


Fig 4B

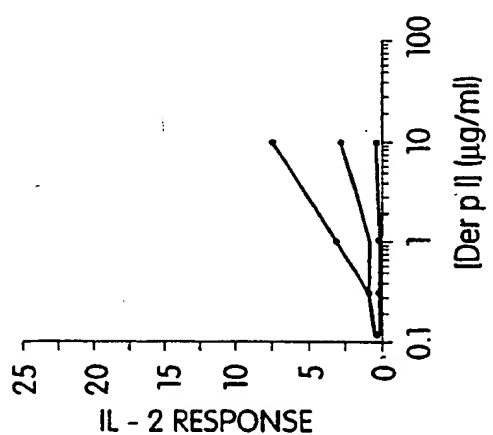


Fig 4F

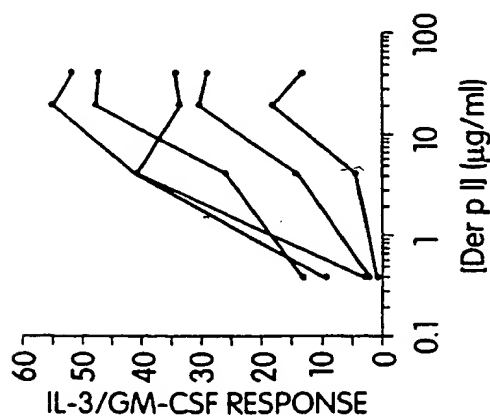


Fig 4A

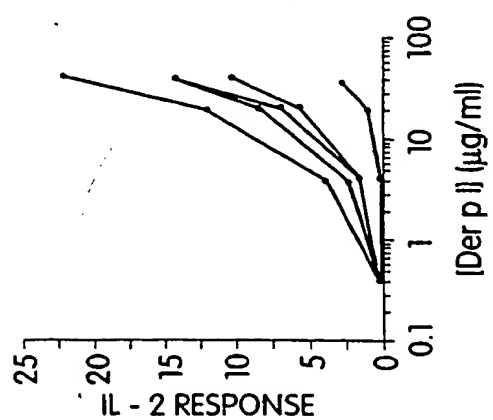


Fig 4E

5/6

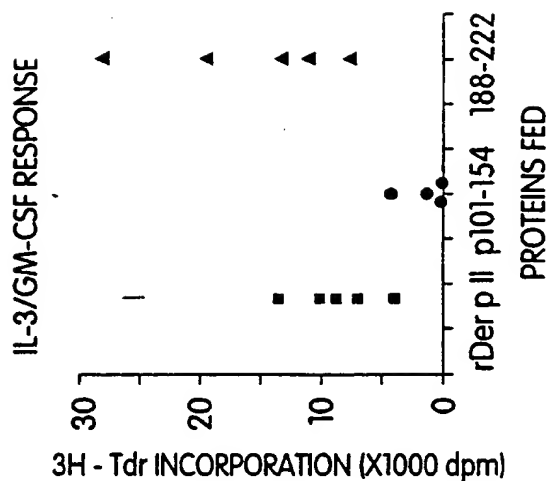


Fig. 5C

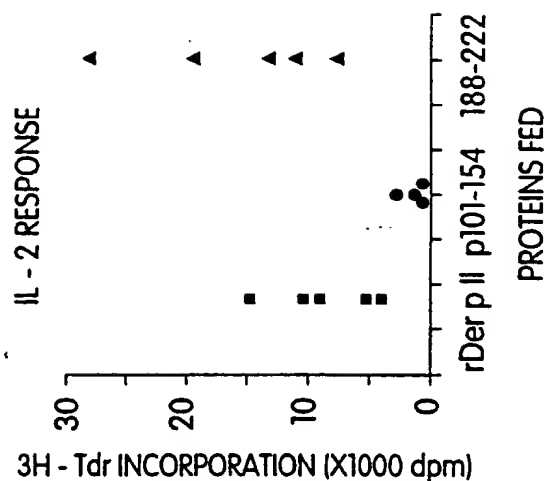


Fig. 5F

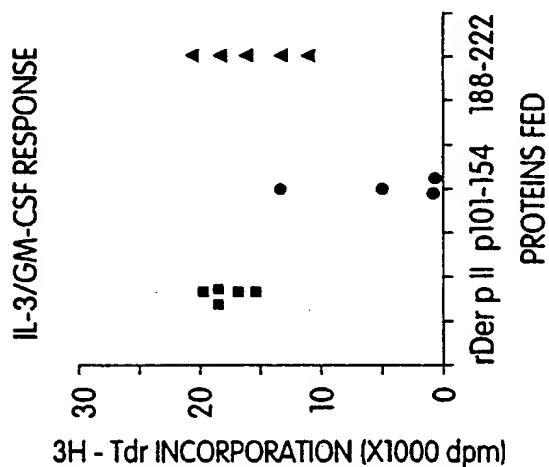


Fig. 5B

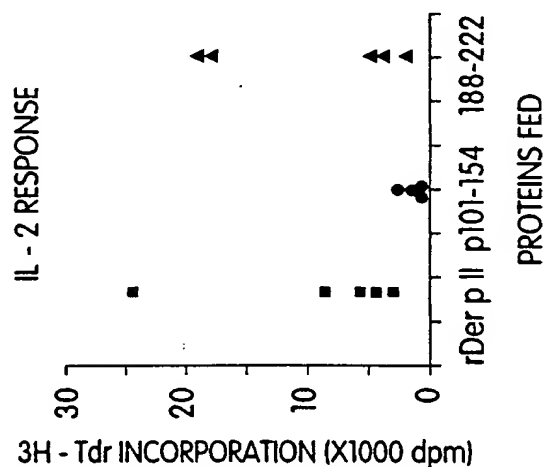


Fig. 5E

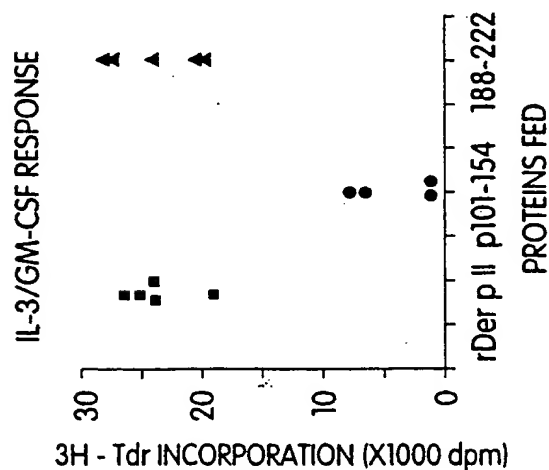


Fig. 5A

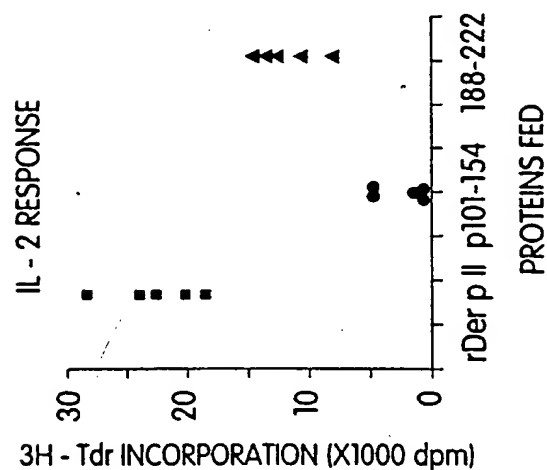


Fig. 5D

6/6

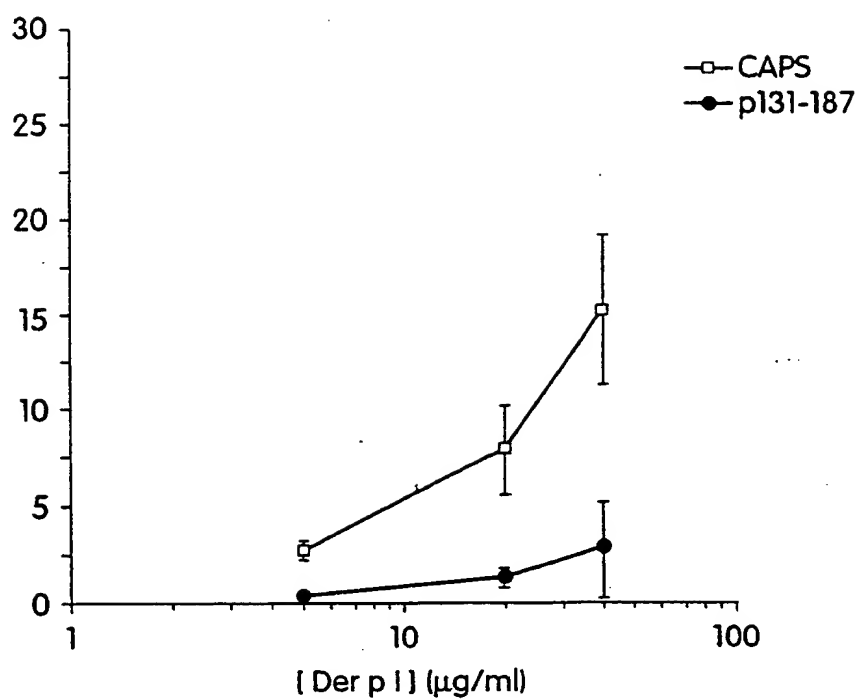


Fig. 6A

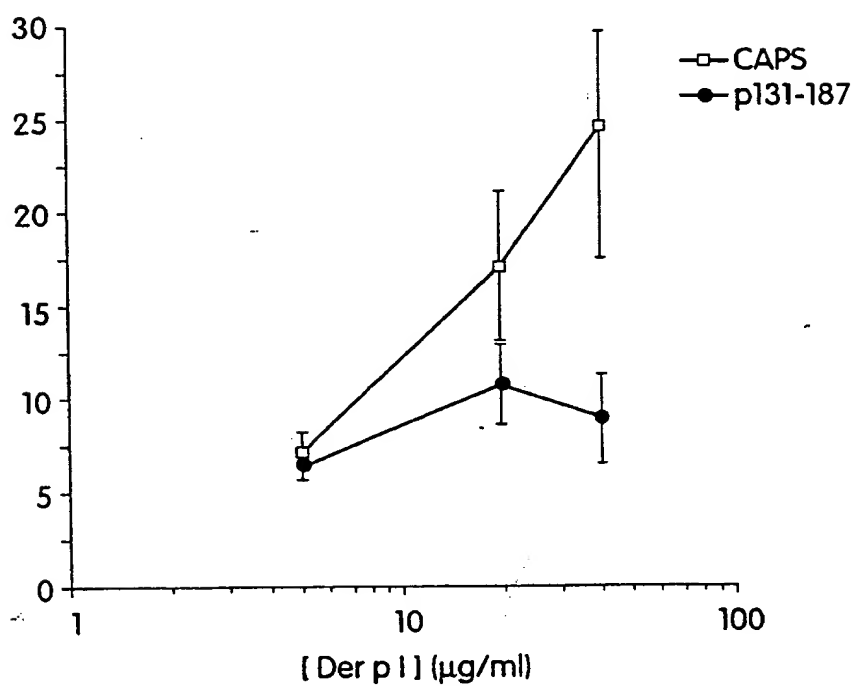



Fig. 6B

A. CLASSIFICATION OF SUBJECT MATTER Int. Cl. ⁵ A61K 39/00, 39/39, 39/35 According to International Patent Classification (IPC) or to both national classification and IPC					
B. FIELDS SEARCHED Minimum documentation searched (classification system followed by classification symbols) IPC ⁵ A61K 39/39, 39/35, 39/00 CHEMICAL ABSTRACTS Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched Electronic data base consulted during the international search (name of data base, and where practicable, search terms used) DERWENT WPAT FILE; CHEMICAL ABSTRACTS CASM FILE KEYWORDS: DER(W)P, DERMATIOPHAGOIDES(W)PTERONYSSINUS, ALLERGEN, PM(W)2, AUTOANTIGEN, TOLERAN., TOLER., INSULIN, MBP, MYELIN (W) BASIC (W) PROTEIN, RH(W) FACTOR: ACETYLCHOLINE (W) RECEPTOR#, GLUTAMIC (W) ACID (W) DECARBOXYLASE, CARBOXYPEPTIDOLASE, THYROID (W) CELL (W) RECEPTOR#, ORGAN (W) TRANSPLANT:					
C. DOCUMENTS CONSIDERED TO BE RELEVANT					
Categ ry*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to Claim N .			
X	Bratanov, M B et al. (1981) Antigen-specific and antireceptor antibodies in the serum of guinea pigs with induced tolerance toward myelin basic protein, Comptes rendus de l'Académie bulgare des Sciences, volume 34, no. 5, pages 713-714, 1981. See whole document.	1-4, 10-12, 19-20, 25-27			
<div style="display: flex; justify-content: space-between;"> <div> <input checked="" type="checkbox"/> Further documents are listed in the continuation of Box C. </div> <div> <input type="checkbox"/> See patent family annex. </div> </div>					
<table style="width: 100%; border: none;"> <tr> <td style="width: 33%; vertical-align: top;"> * Special categories of cited documents : "A" document defining the general state of the art which is not considered to be of particular relevance "E" earlier document but published on or after the international filing date "L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified) "O" document referring to an oral disclosure, use, exhibition or other means "P" document published prior to the international filing date but later than the priority date claimed </td> <td style="width: 33%; vertical-align: top;"> "T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle of theory underlying the invention "X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone "Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art "&" document member of the same patent family </td> <td style="width: 33%;"></td> </tr> </table>			* Special categories of cited documents : "A" document defining the general state of the art which is not considered to be of particular relevance "E" earlier document but published on or after the international filing date "L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified) "O" document referring to an oral disclosure, use, exhibition or other means "P" document published prior to the international filing date but later than the priority date claimed	"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle of theory underlying the invention "X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone "Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art "&" document member of the same patent family	
* Special categories of cited documents : "A" document defining the general state of the art which is not considered to be of particular relevance "E" earlier document but published on or after the international filing date "L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified) "O" document referring to an oral disclosure, use, exhibition or other means "P" document published prior to the international filing date but later than the priority date claimed	"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle of theory underlying the invention "X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone "Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art "&" document member of the same patent family				
Date of the actual completion of the international search 22 August 1994 (22.08.94)		Date of mailing of the international search report 31 AUG 1994 (31.08.94)			
Name and mailing address of the ISA/AU AUSTRALIAN INDUSTRIAL PROPERTY ORGANISATION PO BOX 200 WODEN ACT 2606 AUSTRALIA Facsimile No. 06 2853929		Authorized officer  DAVID HENNESSY Telephone No. (06) 2832255			

C(Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT		
Category*	Citation of document, with indication, where appropriate of the relevant passages	Relevant to Claim No.
X	Lo, D et al (1988) Diabetes and tolerance in transgenic mice expressing class II MHC molecules in pancreatic beta cells, Cell, volume 53, pages 153-168, 8 April, 1988. See introduction; discussion.	
X	Falcioni, F et al (1990) Flexibility of the T cell repertoire - Self tolerance causes a shift of T cell receptor gene usage in response to insulin, J. Exp. Med., volume 171; pages 1665-1681, May 1990. See the discussion in particular.	1-3, 9-12, 19, 25-27
X	Miller, A et al. (1992) Suppressor T cells generated by oral tolerization to myelin basic protein suppress both in vitro and in vivo immune responses by the release of transforming growth factor β after antigen-specific triggering, Proc. Natl. Acad. Sci. USA, volume 89, pages 421-425, January 1992. See the abstract in particular.	1-3, 9-12, 19-20, 25-27
X	Boggs, J M et al. (1992) Stimulation or tolerization of an anti-myelin basic protein T lymphocyte line with membrane fragments from antigen presenting cells, Cellular Immunology, volume 143, pages 23-40, 1992. See the abstract in particular.	1-3, 9-12, 19, 25-27
X	Tan, L et al. (1992) Regulation of the effector stages of experimental autoimmune encephalomyelitis via neuroantigen-specific tolerance induction, The Journal of Immunology, volume 148, pages 2748-2755, 1 May 1992. See the discussion in particular.	1-3, 9-12, 19, 25-27
X	Chemical Abstracts, volume 117, no. 7, 17 August, 1992, page 652, Abstract no. 68258e; Miller, S D et al. (1991) Specific immunoregulation of the induction and effector stages of relapsing EAE via neuroantigen-specific tolerance induction, Ann N. Y. Acad. Sci., pages 79-94.	1-3, 9-12, 19, 25-27
X	AU,A, 87219/91 (THE REGENTS OF THE UNIVERSITY OF CALIFORNIA) 19 March 1992 (19.03.92). See page 7 lines 17-32 in particular.	1-3, 9-12, 19, 25-27
X	AU,A, 87559/91 (RIJKSUNIVERSITEIT TE UTRECHT et al.) 19 March 1992 (19.03.92) See figure 1 in particular.	1-3, 11-12, 19, 27
P,X P,Y	AU,A, 37785/93 (AUTOIMMUNE, INC) 2 September 1993 (02.09.93) see the examples in particular.	1-4, 10-12, 19-20, 25-27 5-9, 11-18, 21-24, 28-29
P,X P,Y	AU,A, 39226/93 (IMMUNOLOGIC PHARMACEUTICAL CORPORATION) 30 September 1993 (30.09.93) see exemplification A in particular.	1-6, 11-14, 17-22, 27-29 7-10, 15-16, 23-26
X Y	AU,A, 20797/88 (BRIGHAM AND WOMEN'S HOSPITAL) 29 December 1988 (29.12.88) see the examples in particular.	1-4, 9-12, 19-20, 25-27 5-9, 11-18, 21-24, 28-29
X Y	AU,A, 69791/91 (BRIGHAM AND WOMEN'S HOSPITAL) 27 June 1991 (27.06.91) see figures 1-8 in particular.	1-4, 10-12, 19-20, 25-27 5-9, 11-18, 21-24, 28-29

C(Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT		
Category*	Citation of document, with indication, where appropriate of the relevant passages	Relevant to Claim No.
X	AU,A, 75789/91 (AUTOIMMUNE, INC) 5 September 1991 (05.09.91) see example 5 in particular	1-4, 10-12, 19-20, 25-27
Y		5-9, 11-18, 21-24, 28-29
X	AU,A, 90237/91 (BRIGHAM AND WOMEN'S HOSPITAL) 30 April 1992 (30.04.92) see examples 15-16 in particular	1-4, 10-12, 19-20, 25-27
Y		5-9, 11-18, 21-24, 28-29
X	AU,A, 19598/88 (PRINCESS MARGARET CHILDREN'S MEDICAL RESEARCH FOUNDATION et al.) 29 December 1988 (29.12.88)	19-24, 27-29
Y		1-9, 11-18

This Annex lists the known "A" publication level patent family members relating to the patent documents cited in the above-mentioned international search report. The Australian Patent Office is in no way liable for these particulars which are merely given for the purpose of information.

Patent Document Cited in Search Report		Patent Family Member					
AU	87219/91	EP	547164	JP	6502916	WO	9204632
AU	87559/91	CA	2071896	EP	503055	IL	99429
		JP	5507501	NZ	239699	WO	9204049
		ZA	9107097				
AU	37785/93	IL	104880	WO	9316724		
AU	39226/93	IL	105153	WO	9319178		
AU	20797/88	DK	6516/89	EP	359783	JP	2503919
		WO	8810120	BR	9203123	CA	2074411
		EP	533223	JP	5196725	NL	9101394
		NO	923161	US	5278564	ZA	9205524
AU	75789/91	BR	9106114	CA	2077340	EP	594607
		HU	9202808	HU	61896	IL	97446
		JP	5508621	NO	923395	WO	9112816
AU	90237/91	CA	2092905	EP	553291	HU	9301089
		IL	99754	JP	5508662	NO	931372
		WO	9206708				
AU	19598/88	EP	362290	JP	3501920	WO	8810297
AU	69791/91	BR	9007950	CA	2070281	EP	506785
		HU	9202072	HU	61487	IL	96734
		JP	5504341	WO	9108760		
END OF ANNEX							